SAM-TR-70-18

EFFECT OF HYPOBARIC ENVIRONMENTS ON THE SUSCEPTIBILITY OF MICE TO BACTERIAL TOXINS

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April 1970

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FOREWORD

This work was done in the Microbiology-Immunology Branch under task No. 775809 during 1969. The paper was submitted for publication on 19 January 1970.

The author expresses appreciation to the chamber crews and technicians of the Infectious Diseases Branch for providing the hypobaric environments, and to Alton J. Rahe, Biometrics Division, for the statistical analyses.

The animals involved in this study were maintained in accordance with the "Guide for Laboratory Animal Facilities and Care" as published by the National Academy of Sciences–National Research Council.

This report has been reviewed and is approved.

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ABSTRACT

Two toxin-producing bacteria, *Staphylococcus* spp. and *Salmonella enteritidis*, affect man and other animals. Staphylococci produce enterotoxin of the "B" type (SEB) that is responsible for food poisoning. *S. enteritidis* produces a lipopolysaccharide (LPS) that is both pyrogenic and toxic for humans. SEB and LPS together act as synergists in mice. Neither toxin, separately, is markedly lethal. Injected in sequence, they cause substantial lethality.

Twenty-one experiments were conducted at 27,000 ft. simulated altitude with varying gaseous environments and temporal sequences of SEB- and LPS injections in mice. These studies established that hypobaric environments decreased the susceptibility of mice to the lethal effects of the SEB-LPS combination when the animals remained at altitude. This was true whether the mice were acclimatized or not and regardless of the gaseous composition. Greatest resistance or least susceptibility to toxins was demonstrated at 27,000 ft. simulated altitude with 50% O_2 – 50% N_2.

It is recognized that this decrease in susceptibility may be mediated by increased levels of adrenal activity associated with the stress. In this regard, those environments that were most stressing favored mouse survival. Additional experiments, however, involving injection of hydrocortisone or corticosterone did not decrease susceptibility. As an explanation of the results, there remains the possibility that alterations in the oxygen tensions at the cellular level due to the gaseous environments may also favor a biochemical or metabolic environment that is more protective than ground conditions.

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I. INTRODUCTION

Bacterial products may directly influence homeostatic balance and indirectly affect the ability of man to perform optimally in demanding aerospace tasks. For example, certain bacteria produce staphylococcal enterotoxin (SE). This toxin is responsible in man for incidents of food poisoning with typical signs of nausea, violent repeated emesis, and diarrhea (1).

Many gram-negative bacteria produce another toxin, lipopolysaccharide endotoxin (LPS), that is both pyrogenic and toxic in humans (2).

When, or if, certain concentrations of SE and LPS appear in mice, the toxins exert their effects by a synergistic action (3). For example, mice show no visible effect from an intraperitoneal injection of SE (200 μg.)—a dosage that is at least ten times greater than the emetic dose for man. Furthermore, LPS injections (150 μg.) into mice cause only transient incapacitation, but an SE injection followed 4 hours later by LPS is a lethal combination.

It is possible that effective concentrations of these toxins could occur in men under simulated or actual hypobaric environments. For this reason, it is pertinent to determine whether such conditions precipitate, enhance, or attenuate their effects. Using lethality in mice as the criterion in a model system does not necessarily imply that extrapolation to man is valid. However, since SE and LPS, separately, do affect humans, the experiments undertaken provide considerations for anticipating untoward conditions in human subjects exposed to hypobarism and altered gas environments.

II. MATERIALS AND METHODS

Toxins

The SE was a lyophilized extract predominantly of the "B" type enterotoxin, generally referred to as SEB (4). Appropriate amounts were solubilized in phosphate-buffered saline (PBS, pH 6.8 to 7.0) and stored at −20° C. in sealed ampules containing 500 μg./ml. or 100 μg./ml. Several concentrations of the SEB were separately titrated by intraperitoneal injections into mice followed by either of two LPS levels to arrive at the optimum combination for studying increased or decreased percent mortality. The optimum combination was 12 μg./0.1 ml. of SEB followed 4 hours later by 150 μg./0.1 ml. of LPS. These were the concentrations used throughout the study.

LPS was a commercially prepared product extracted and lyophilized from the gram-negative organism, *Salmonella enteritidis*. One concentration, 1.5 mg./ml., was prepared by solubilization in PBS and used when required for the second intraperitoneal injection.

Animals and housing

The Swiss-Webster strain of mice was used for all experiments. Males, weighing 20 to

35 gm., were housed in groups of 5 in plastic mouse cages (28 cm. x 18 cm. x 13 cm.) with perforated metal lids and a pellet trough. Food and water were provided ad libitum. The sawdust bedding was changed weekly. Most experiments had two groups of 20 animals for each of the toxin or exposure conditions. These two groups were considered as duplicate experiments conducted during the same chamber exposure to validate the reproducibility of the results for any one set of conditions under investigation.

Hypobaric conditions

Decreased barometric pressure equivalent to approximately 27,000 ft. (258 mm. Hg) simulated altitude was imposed on groups of mice as shown in table I. The simulated flight profile was achieved by ascending 3,000 ft./min. up to 8,000 ft., holding for 5 minutes; then ascending in steps of 2,000 ft./min. and holding for 5 minutes at each step. Chamber temperature was maintained at approximately 21° C.

Injection times and mortality counts

All injections were given intraperitoneally. The initial injection, SEB or sterile saline, was given between 8:00 and 9:00 a.m. to minimize circadian variability (5). The second injection, LPS or sterile saline, was always given between 1:00 and 2:00 p.m. In order to compare mortality rate as well as total mortality among the various conditions, mortality census was taken at 20, 25, 44, and 50 hours after the second injection. These counts were always made, in appropriate cases, at the simulated flight conditions.

III. RESULTS

The data indicating deaths at each sampling time (e.g., 20, 25, 44, and 50 hours after last injection) were analyzed separately. The variable analyzed was the proportion (p) of total deaths that occurred for each group for the particular time. The distribution of the p values is binomial in form where the variance is dependent upon the value of p. In order to analyze these data using an analysis of variance procedure, an arc sine $\sqrt{p}$ transformed variable was obtained (6). This transformed variable has a theoretical constant variance for all p values—a requirement for the analysis of variance procedure. It should be emphasized that the results from the different observation times are not independently graphed in the figures. For example, 3 deaths at the 25-hour sampling time were added to 2 more at the 44-hour time to plot a mortality of 5 at the latter reading. Except when otherwise indicated, two groups of 20 mice each were used for each treatment. These groups comprised duplicate experiments exposed simultaneously. Considering all duplicates, the average difference in deaths at the 50-hour reading time was 1.3 mice. The variability between duplicate groups at each treatment provided the error term for testing among treatments. For purposes of graphing in figures 1 to 6, the appropriate duplicate experiments of two groups of 20 each were combined.

The hypotheses for these experiments were based upon certain questions regarding the effects of hypobaric conditions, and the experiments were designed to answer these questions. Obviously, every possible combination of the 21 experiments might be considered. This would be extremely complex and, in some instances, not pertinent. For this reason, each question is considered and the appropriate results indicated below.

Question 1. Does the length of stay at altitude (acclimatization) at 100% oxygen before exposure to SEB and LPS affect total deaths up to 50 hours postinjection?

Figure 1 compares experiments in which the animals were returned to ground level after acclimatization to altitude for either 48 hours (exp. 1) or 7 days (exp. 14) at 100% oxygen. The toxins were injected at ground level after altitude. As indicated, statistical significance was obtained at the 44-hour reading time, indicating a decrease in deaths when mice were acclimatized for 7 days.

Also in figure 1, a significant retardation in deaths was observed ($P < .05$) at 25 hours

TABLE I

Experiment variables

Exp. No.	Atmosphere	Duration of acclimatization	Environment of SEB injection	Environment of LPS injection	Remarks
1	100% O_2	48 hr.	Ground level	Ground level	Toxins immediately postaltitude
2					Toxins 24 hr. postaltitude
3					Toxins 48 hr. postaltitude
4	Normal	—	Ground level	Ground level	Ground controls; saline only
5			—	Ground level	Saline in lieu of SEB; ground control
6	50% O_2–50% N_2	48 hr.	Altitude	Altitude	Remained at altitude
7			—	Altitude	Saline in lieu of SEB (alt.); remained at altitude*
8			Altitude	—	Saline in lieu of LPS (alt.); remained at altitude*
9			—	—	Saline in lieu of SEB (alt.) and LPS (alt.); altitude control*
10	.0% O_2–30% N_2	48 hr.	Altitude	Altitude	Remained at altitude
11			—	Altitude	Saline in lieu of SEB (alt.); remained at altitude†
12	100% O_2	None	Altitude	Altitude	Remained at altitude
13		7 days	Altitude	Altitude	Remained at altitude
14		7 days	Ground level	Ground level	Toxins immediately postaltitude
15		48 hr.	Altitude	Altitude	Remained at altitude
16		48 hr.	Altitude	—	Saline in lieu of LPS (alt.); remained at altitude
17		48 hr.	—	Altitude	Saline for SEB (alt.); remained at altitude
18		48 hr.	—	—	Saline in lieu of SEB (alt.) and LPS (alt.); altitude control†
19	70% O_2–30% N_2	None	Ground level	Altitude	Remained at altitude
20			—	Altitude	Saline for SEB (GL); remained at altitude
21		None	Ground level	—	Saline for LPS (GL); remained at altitude

*N = 10, instead of usual 20 mice per group.

†Only 1 group of 20 mice tested, instead of the usual 2 groups.

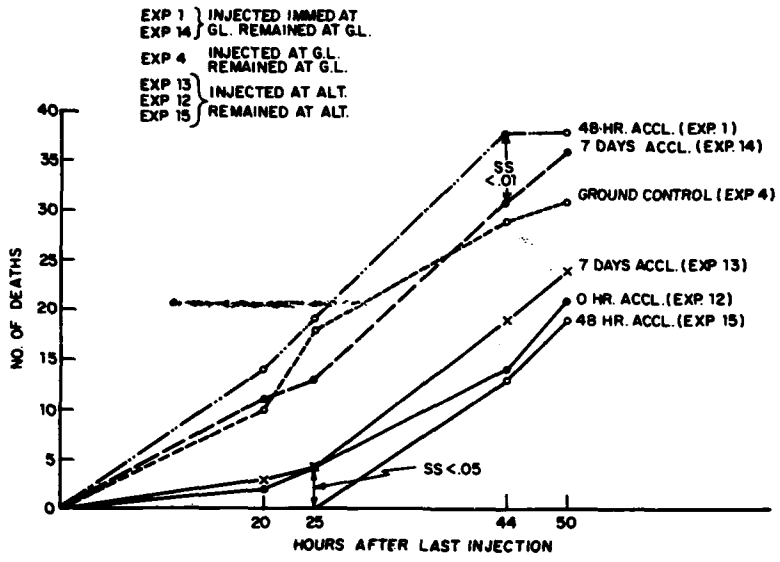

FIGURE 1

Number of deaths vs. hours after LPS injection for mice exposed to SEB and LPS toxins plus simulated altitude of 27,000 ft. at 100% O_2. SS means statistically significant at the probability levels shown. See also table II.

for animals acclimatized to altitude for 48 hours (exp. 15) compared to those receiving no acclimatization (exp. 12) or 7-day acclimatization (exp. 13). In these experiments the animals were injected with toxins at the simulated altitude following the stated acclimatization period.

Question 2. Does exposure to 100% oxygen at a simulated altitude of 27,000 ft. alter the susceptibility of mice to the SEB-LPS combination?

This question concerns all possible comparisons of experiments shown in figure 1. Baseline for such comparisons was the curve for those animals that were injected at ground level and that remained at ground level (exp. 4). Figure 1 shows that the mortality curves for animals remaining at altitude, whether acclimatized (exps. 13 and 15) or not acclimatized (exp. 12), were uniformly lower than the mortality curve for the ground controls (exp. 4). The animals in experiment 12 were injected immediately upon attaining altitude and did not show significant retardation in mortality compared to acclimatized mice.

Table II is provided to indicate at what reading times statistical significance in mortality was found for both questions 1 and 2.

Question 3. Does exposure to altitude at 70% oxygen–30% nitrogen, with or without acclimatization, affect the susceptibility of mice to SEB and LPS given in sequence?

Figure 2 shows curves comparing the ground controls (exp. 4) with acclimatized (exp. 10) and unacclimatized (exp. 19) animals along with the probability levels for the results at the 20-hour and 25-hour readings. In these comparisons, significance was reached at only

TABLE II

Probability levels for mice mortality from SEB-LPS injections based on duration of acclimatization to altitude

Experiment	Duration of acclimatization	Reading time (hrs.)			
		20	25	44	50
12	0	$<.01$	$<.01$	$<.01$	$<.05$
15	48 hr.	$<.01$	$<.01$	$<.01$	$<.05$
13	7 days	$<.01$	$<.01$	$<.05$	NS
1	48 hr.	NS	NS	$<.01$	$<.05$
14	7 days	NS	NS	NS	NS

Experiments were conducted at 27,000 ft. simulated altitude, 100% oxygen. Experimental groups were injected with SEB followed 4 hours later by LPS. Probabilities are based on baseline for experiment 4.

NS = Not statistically significant.

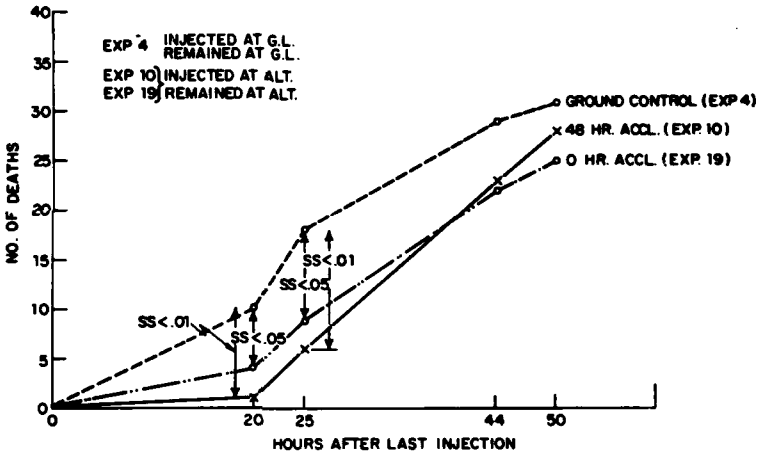

FIGURE 2

Number of deaths vs. hours after LPS injection for mice exposed to SEB and LPS toxins plus simulated altitude of 27,000 ft. at 70% O_2–30% N_2. SS means statistically significant at the probability levels shown.

2 of the 4 reading times; whereas comparisons of ground controls and similar altitude exposures at 100% oxygen showed significance was achieved at 4 out of 4 reading times. With either atmosphere (100% oxygen, or 70% oxygen–30% nitrogen), retardation of death was evident compared to ground-level animals.

Question 4. Does the gas composition influence the SEB-LPS effect on mice that were acclimatized for 48 hours and remained at altitude?

The number of mice that died at three atmospheric mixtures are shown in figure 3.

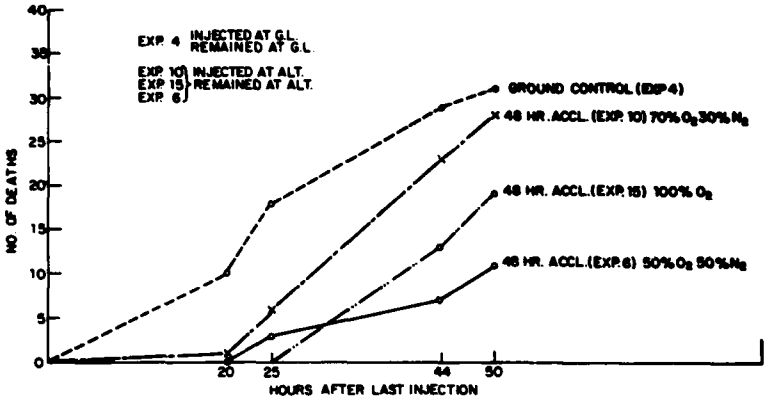

FIGURE 3

Number of deaths vs. hours after LPS injection for mice exposed to SEB and LPS toxins plus simulated altitude of 27,000 ft. at three different atmospheres. See also table III.

TABLE III

Probability levels for mice mortality from SEB-LPS injections combined with hypobarism under three different gas mixtures

Experiment	Gas mixture	Reading times (hrs.)			
		20	25	44	50
6	50% O_2 – 50% N_2	< .01	< .01	< .01	< .01
10	70% O_2 – 30% N_2	< .01	< .01	NS	NS
15	100% O_2	< .01	< .01	< .01	NS

Experiments were conducted at 27,000 ft. simulated altitude. All three experimental groups received 48-hour acclimatization to altitude, were injected at altitude, and remained at altitude. Probabilities are based on baseline for experiment 4.

NS = Not statistically significant.

Table III shows the significant differences in probability levels at the various recording times. As indicated, the smallest number of deaths out of two groups of 20 mice each and the greatest inhibition of death rate was at 50% oxygen–50% nitrogen. Note that when considering mixed atmospheres at 27,000 ft. (258 mm. Hg), the gaseous environment with the lowest partial pressure of oxygen (50% oxygen, 129 mm. Hg) at 50 hours' exposure showed the greatest protection from toxin death when compared to 70% oxyge. (180 mm. Hg partial pressure) or 100% oxygen.

Question 5. Does simulated altitude for 48 hours at 100% oxygen have any effect on the susceptibility of mice to the SEB-LPS combination?

Data from experiments 1, 2, 3, and 4 (ground control) are plotted in figure 4 for

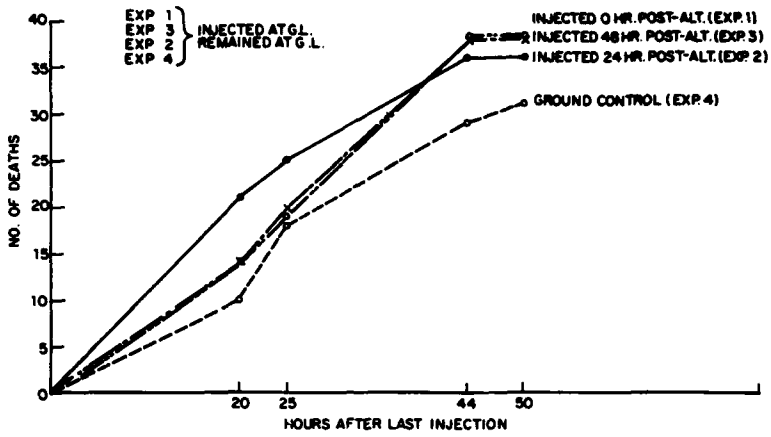

FIGURE 4

Number of deaths vs. hours after LPS injection for mice exposed to SEB and LPS toxins plus simulated altitude of 27,000 ft. at 100% O_2. See also table IV.

TABLE IV

Probability levels for effect of hypobarism on mice mortality from SEB-LPS injections

Experiment	Time of SEB injection	Reading time (hrs.)			
		20	25	44	50
1	Immed. postaltitude	NS	NS	$<.01$	$<.05$
2	24 hr. postaltitude	$<.01$	NS	NS	NS
3	48 hr. postaltitude	NS	NS	$<.01$	$<.05$

Experiments were conducted at 27,000 ft. simulated altitude, 100% oxygen. All experimental animals received 48-hr. acclimatization to altitude, were injected at ground level, and remained at ground level following injections. Probabilities are based on baseline for experiment 4.

NS = Not statistically significant.

comparison. Statistical significances are tabulated separately in table IV. Increases in mortality over ground controls were apparent for mice injected with toxins immediately after, or 48 hours after, simulated altitude, and accelerated deaths were observed for animals injected 24 hours after returning from decreased pressure. It should be noted also that these mortality curves are all steeper than the curve for the ground control group. When the animals remained at altitude after injection, the mortality curves were less steep (cf. figs. 1-3).

Question 6. Do SEB and LPS show a synergistic action in mice acclimatized at 100% oxygen for 48 hours and remaining at altitude after toxin injections?

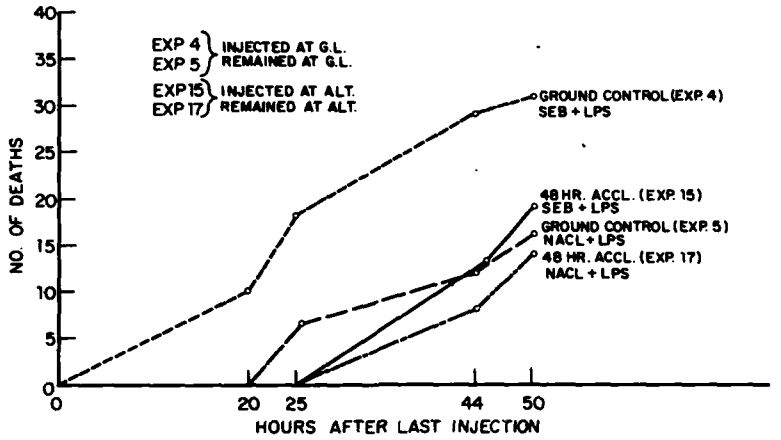

FIGURE 5

Number of deaths vs. hours after LPS injection for mice exposed to SEB and LPS, or saline and LPS, plus simulated altitude of 27,000 ft. at 100% O_2.

As shown in figure 5, the SEB-LPS combination acts synergistically at ground level (exp. 4 vs. exp. 5); however, when acclimatized to altitude, the susceptibility of the animals to the SEB and LPS injections decreases. The results for the two toxins are not significantly different from those for one toxin (exp. 15 vs. exp. 17). No deaths resulted in groups injected with SEB followed by saline, or two injections of saline only; therefore, these results were not plotted.

The question of synergism of SEB-LPS at 70% oxygen–30% nitrogen was also explored in animals that were not acclimatized (exps. 19, 20, and 21) and that remained at altitude (fig. 6). Under this environment, the death curve for mice given both SEB and LPS before altitude (exp. 19) was not significantly different from that of the ground controls (exp. 4). Both of these curves, however, were significantly different ($P < .01$) at the 44-hour and 50-hour observation times from groups injected with only one toxin (exps. 20 and 21).

In experiments 6 to 9, the gas mixture was 50% oxygen–50% nitrogen, the animals were acclimatized for 48 hours prior to injection, and the animals remained at altitude after toxin exposure. The mortality rate of the SEB-LPS combination was greater than that for either toxin alone ($P < .01$).

Marked significance in mortality ($P < .01$) was seen for experiments with saline followed by LPS treatment as between experiments with 48-hour acclimatization at 100% oxygen (exp. 17) and those with 50% oxygen–50% nitrogen (exp. 7) at both the 44-hour and 50-hour reading times. At the 50% oxygen concentration, the mortality was considerably less. The difference between 50% and 70% oxygen conditions was not statistically significant.

SEB followed by saline was not lethal at ground level. Among experiments with the three gas compositions, the mortality was zero at all of the recording times when SEB followed by saline was considered.

IV. DISCUSSION

There are two general questions that these data may answer: (1) Do hypobaric conditions with associated gaseous environments alter the susceptibility of mice to SEB and LPS, alone

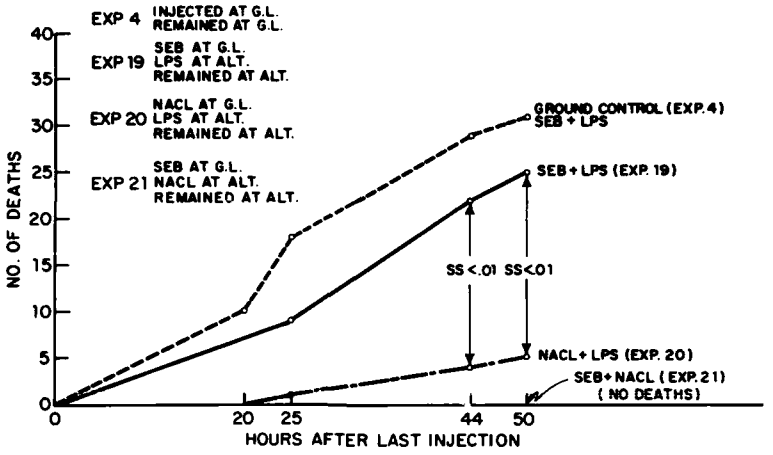

FIGURE 6

Number of deaths vs. hours after LPS injection for mice exposed to SEB, LPS, or SEB plus LPS at a simulated altitude of 27,000 ft., 70% O_2–30% N_2. These mice were not acclimatized.

or in combination? (2) What hypobaric environments would appear to be most stressing when susceptibility to these toxins is used as the biologic indicator?

A comparison of figures 1 to 6 shows that hypobaric environments do decrease the susceptibility of mice to the SEB-LPS injections. In experiments in which the mice remained at altitude after the injections, decreased susceptibility was observed. This was generally true whether the animals were acclimatized or not, and regardless of the gaseous composition. There would seem to be a common stress effect produced by hypobarism. Superimposed upon this may be other in vivo quantitative or qualitative biochemical changes induced by the gaseous composition. Simulated altitude and the changed composition of the respiratory air are stresses in the sense that they require adjustments by the animal to survive. It is apparent that in several comparisons statistically significant differences between conditions were observed only at certain times. There are numerous dynamic changes and interchanges taking place among the metabolic processes and the gas-cellular environment during hypobaric exposure. These exchanges logically occur at certain times and either inhibit or enhance the direct or indirect effects of the injected toxins.

A direct association exists between stress and the stimulation of adrenal cortical activity in animals (7, 8). Furthermore, a positive correlation has been reported between stress and resistance to foreign substances (9-11). Undoubtedly, the actual situation in hypobaric environments is considerably more complex than an increase in steroid levels since neither three injections of hydrocortisone nor three injections of corticosterone at two concentrations on three successive days decreased the susceptibility of mice to the SEB-LPS combination (12).

From these facts, the changes in susceptibility of mice to the lethal action of the SEB-LPS toxins would seem to be a measure of the severity of stress. For example, altitude chamber conditions that were the most stressing provoked the greatest resistance to the toxins. On this basis, remaining at altitude—whether acclimatized for 48 hours or 7 days—

is a stressing situation for mice. Especially stressing is hypobarism combined with 50% oxygen–50% nitrogen.

Applying these results to flying personnel, these data indicate that during hypobarism and concurrent flight atmospheres, higher doses of these fever- and emesis-inducing bacterial toxins are probably required to produce an incapacitating effect. When such effect is manifested, larger than usual doses of therapeutic drugs should be considered.

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12. Glenn, W. G. Unpublished observations.

UNCLASSIFIED

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) USAF School of Aerospace Medicine Aerospace Medical Division (AFSC) Brooks Air Force Base, Texas	2a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED 2b. GROUP

3. REPORT TITLE

EFFECT OF HYPOBARIC ENVIRONMENTS ON THE SUSCEPTIBILITY OF MICE TO BACTERIAL TOXINS

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Progress report January - May 1969

5. AUTHOR(S) (First name, middle initial, last name)

William G. Glenn

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
April 1970	10	12

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 7753	SAM-TR-70-18
c. Task No. 7753 09	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10. DISTRIBUTION STATEMENT

This document has been approved for public release and sale; its distribution is unlimited.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	USAF School of Aerospace Medicine Aerospace Medical Division (AFSC) Brooks Air Force Base, Texas.

13. ABSTRACT

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It was recognized that this decrease in susceptibility may be mediated by increased levels of adrenal activity associated with the stress. In this regard, those environments that were most stressing favored mouse survival. Additional experiments, however, involving injection of hydrocortisone or corticosterone did not decrease susceptibility. As an explanation of the results, there remains the possibility that alterations in the oxygen tensions at the cellular level due to the gaseous environments may also favor a biochemical or metabolic environment that is more protective than ground conditions.

DD FORM 1 NOV 68 1473

UNCLASSIFIED

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14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Immunology						
Microbiology						
Toxicology						
Altitude, bacterial toxins						
Toxins, simulated altitude						
Bacterial toxins						

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